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Note

High-performance liquid chromatographic determination of dihydralazine in human plasma

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Dihydralazine (1,4-dihydrazinophthalazine)* is a vasodilator anti-hypertensive drug reported to be particularly useful in the treatment of renal and idiopathic forms of hypertension[1–3]. It is closely related to the monohydrazine-substituted phthalazine and anti-hypertensive agent hydralazine[3–5]. The pharmacokinetics of dihydralazine in man do not appear to have been fully investigated, presumably because a suitably sensitive analytical method has not been available.

Several methods have been published for the determination of hydralazine in plasma, which, in theory, should be directly applicable to the determination of dihydralazine. Methods utilizing hydrazone formation with *p*-hydroxybenzaldehyde or *p*-methoxybenzaldehyde have been published[6–8] but such methods are not sufficiently sensitive to measure plasma concentrations of the drug obtained after single oral doses at therapeutic levels.

Like hydralazine, dihydralazine is unstable at alkaline pH and cannot be extracted by organic solvents at acid pH. Druey and Ringier[9] showed that hydralazine reacts with nitrous acid to form tetrazolophthalazine, which is stable at alkaline pH and can be extracted from aqueous solutions with benzene. A gas chromatographic procedure based on this derivative formation has been published[10]. Dihydralazine reacts with sodium nitrite at acidic pH to yield a disubstituted derivative, 1,4-ditetrazolophthalazine, which, although extractable with benzene from biological materials at alkaline pH, appears to be unstable under the gas chromatographic conditions reported for hydralazine.

High-performance liquid chromatography is regarded as a useful technique for the measurement of heat-labile compounds and under appropriate conditions has a sensitivity similar to that of gas chromatography. This paper describes the use of high-performance liquid chromatography (HPLC) in a reversed-phase mode to separate and measure dihydralazine in human plasma samples with good accuracy, precision and sensitivity. The structurally related compound 1-hydrazino-4-methylphthalazine (methylhydralazine), which also forms a tetrazolo derivative with sodium nitrite, was employed as the internal standard (Fig. 1). The method could also be adapted for the measurement of hydralazine in plasma.

* Present in Pertenso, produced by Melusin Schwarz-Monheim GmbH, Monheim, G.F.R.

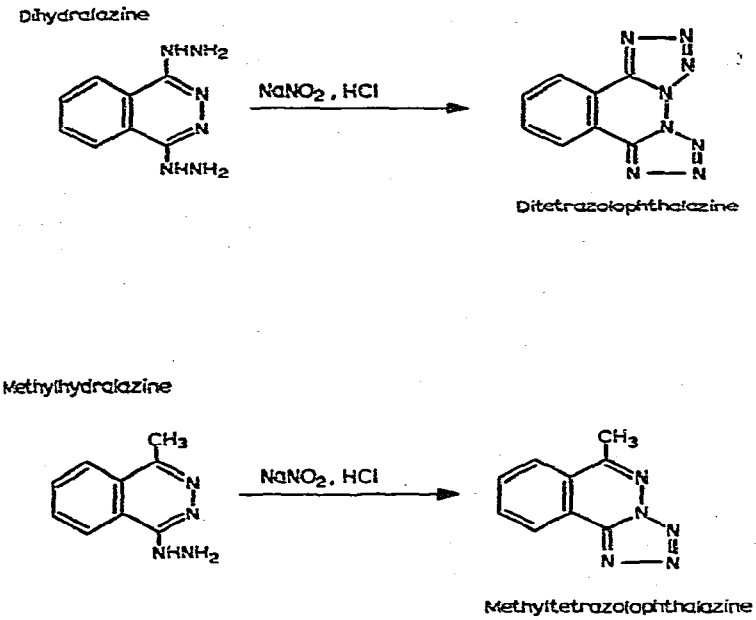

Fig. 1. Formation of tetrazolo derivatives of dihydralazine and the internal standard methylhydralazine.

EXPERIMENTAL

Materials

Reagents were of analytical grade, and inorganic reagents were prepared in glass-distilled water. Benzene (Nanograde, Mallinckrodt, St. Louis, Mo., U.S.A.) was obtained from Camlab (Cambridge, Great Britain) and was not further purified. Methanol (HPLC grade) was supplied by Fisons Scientific Supplies (Loughborough, Great Britain). Standard solutions of dihydralazine (10 mg/ml) and methylhydralazine (10 mg/ml) were freshly prepared and diluted in 2 *M* hydrochloric acid to provide working standards (10 ng/μl) immediately prior to their derivatization. Dihydralazine (Batch No. WE 860) and methylhydralazine suitable for use as analytical standards were kindly provided by Dr. R. Bonn of Sanol Schwarz-Monheim GmbH, Monheim, G.F.R.

Extraction

Derivatization and extraction of the tetrazolo derivative of dihydralazine was carried out in glass 30-ml stoppered test-tubes. Plasma samples (2 ml) were spiked with the internal standard methylhydralazine (40 ng/ml) and mixed with 2 *M* hydrochloric acid (2 ml) and distilled water (2 ml) followed by 50% (w/v) sodium nitrite solution (200 μl). The addition of water was necessary to prevent excessive frothing during derivatization. The mixture was allowed to stand at room temperature for 15 min and was then adjusted to pH 10 with 2.5 *M* sodium hydroxide solution. The tetrazolo derivatives which had formed were then extracted by shaking the reaction mixture with benzene (10 ml) for 5 min. The organic phase was removed and

evaporated to dryness under a stream of dry nitrogen at 37°. The dry residue was re-dissolved in 100 μl of methanol (HPLC grade) and a portion (50–70 μl) injected into the chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M 6000A pump (Waters Assoc., Northwich, Great Britain) linked to a Perkin-Elmer LC 55 variable-wavelength ultraviolet detector (Perkin-Elmer, Beaconsfield, Great Britain) operated at 230 nm at a sensitivity of 0.002 a.u.f.s. Injection was made by syringe via a UK6 universal injector (Waters Assoc.). The column was 25 cm × 4.6 mm I.D., pre-packed with Partisil 25 ODS 2 (a C_{18} hydrocarbon bonded to a 10-μm support; Whatman Ltd., Maidstone, Great Britain). Chromatography was performed in a reversed-phase mode using a solvent system of 60% methanol in 0.01 *M* potassium dihydrogen orthophosphate (adjusted to pH 3.0 with orthophosphoric acid), at a flow-rate of 2 ml/min, which resulted in a column pressure of approximately 2000 p.s.i. Under these conditions, ditetrazolophthalazine and methyltetrazolophthalazine were eluted with retention times of 6.0 and 3.5 min, respectively (Fig. 2). The tetrazolo derivative of hydralazine was eluted with a retention time of 2.5 min and thus did not interfere.

Mass spectrometry

The structure of the ditetrazolo derivative was examined by mass spectrometry

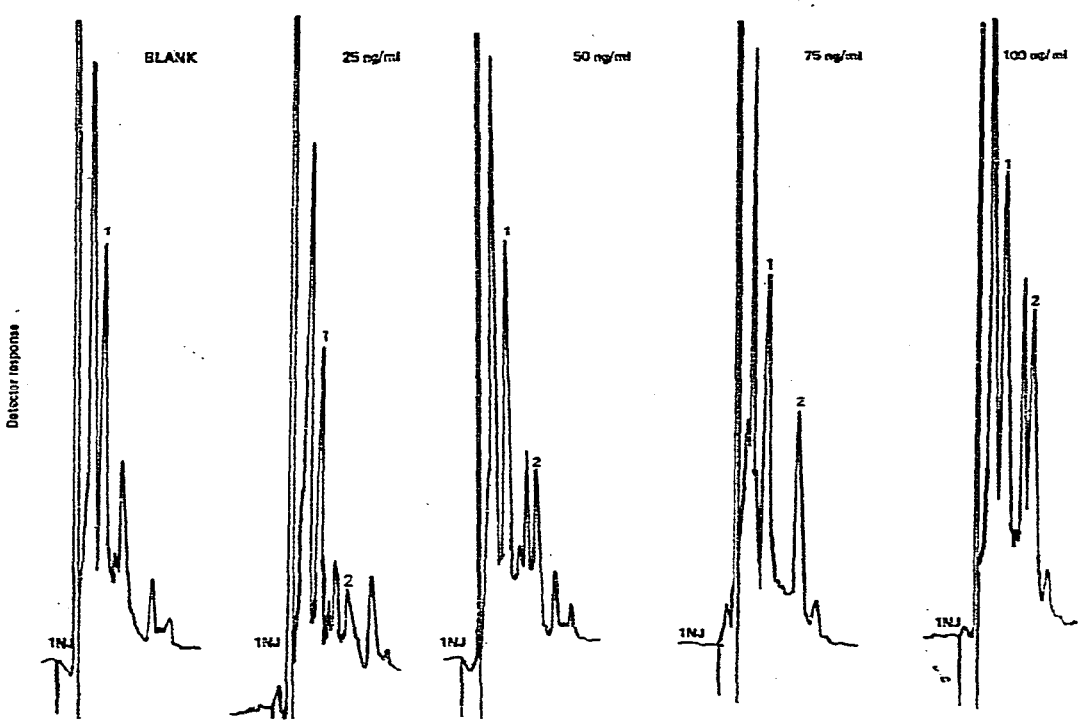

Fig. 2. High-performance liquid chromatograms of human plasma spiked with 0, 25, 50, 75 or 100 ng of dihydralazine (peak 2). The internal standard methylhydralazine (peak 1) was spiked at a concentration of 40 ng/ml.

using a Micromass 16 F mass spectrometer (V.G. Organic, Altrincham, Great Britain) operated in either the electron impact or chemical ionization (reagent gas, isobutane) modes. An electron beam energy of 70 and 50 eV and an emission current of 100 and 200 μA were employed, respectively. Samples were introduced by the direct insertion probe.

The mass spectrum of the ditetrazolo derivative, obtained using the chemical ionization mode, showed a peak at *m/e* 213 corresponding to the molecular ion (M + 1), and also a peak at *m/e* 129 corresponding to dicyanobenzene, an expected fragment. Dicyanobenzene was the only notable fragment present (*m/e* 128) after electron impact mass spectrometry and the molecular ion (*m/e* 212) was absent. The ease of decomposition of ditetrazolophthalazine to dicyanobenzene was the probable cause of its instability observed during gas chromatography.

Collection of samples

Blood samples were withdrawn into heparinized tubes from two volunteer male human subjects who had received single oral doses of 20 mg of dihydralazine sulphate as a suspension in water. The samples were centrifuged immediately and the plasma was subjected to the derivatization procedure within 15 min of collection. Once the dihydralazine in plasma had been derivatized and extracted, the resulting extracts were stable at −20°.

RESULTS AND DISCUSSION

Concentrations of dihydralazine in plasma were determined from calibration graphs constructed by plotting the ratio of peak-height measurements of drug (as ditetrazolophthalazine) to the internal standard methylhydralazine (40 ng/ml as methyltetrazolophthalazine) added to plasma over the concentration range 0–100 ng/ml (Fig. 3). The recovery of derivatized dihydralazine from plasma was identical with that from water, although the absolute recovery could not be determined because reference standard tetrazolo derivatives were not available. Plots of peak-height ratios (Y) against dihydralazine concentrations (X) were linear, $Y = 0.0028 + 0.0077X$ ($r = 0.994$). The 95% confidence limits of taking peak-height ratios as an estimate of plasma concentrations of dihydralazine were ± 80% at 10 ng/ml, ± 16% at 50 ng/ml and ± 9% at 100 ng/ml. The precision of the method for the measurement of plasma concentrations of dihydralazine, determined as the coefficient of variation of the mean of five replicate assays, was ± 14.3, ± 8.1, ± 4.4 and ± 4.6% at 10, 25, 50 and 100 ng/ml, respectively.

None of the pre-dose plasma samples examined contained any components which were eluted with retention times the same as those of either dihydralazine or the methylhydralazine derivatives. Therefore, the limit of detection of the method was taken as 2.5 ng/ml, allowing a signal-to-noise ratio of 4:1. The method was applied to plasma samples obtained from human subjects dosed with dihydralazine. The results showed that plasma concentrations of dihydralazine increased rapidly to reach peak values of 64.5 and 49.1 ng/ml after 1 h in the two subjects (Table I). Plasma concentrations then declined rapidly in the plasma of each subject with an apparent half-life of about 4 h.

This HPLC method, after minor modifications to the chromatographic solvent

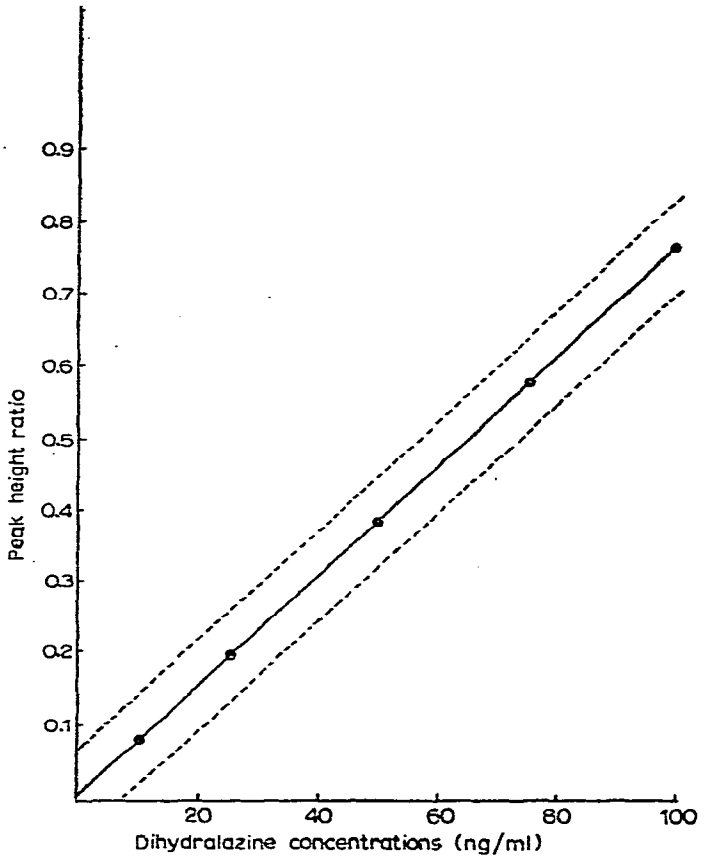

Fig. 3. Standard graph for the measurement of dihydralazine in plasma, constructed by plotting peak-height ratio measurements of derivatized dihydralazine to internal standard against concentration of dihydralazine. The broken lines correspond to the 95% confidence limits of the curve.

TABLE I

MEAN PLASMA CONCENTRATIONS OF DIHYDRALAZINE AFTER SINGLE ORAL DOSES OF 20 mg OF DIHYDRALAZINE SULPHATE TO HUMAN SUBJECTS

Time (h)	*Concentration (ng/ml)*	
	Subject A	*Subject B*
Pre-dose	< 2.5	< 2.5
0.5	51.4	22.8
1	64.5	49.1
1.5	49.0	28.9
2	44.7	26.0
3	22.9	21.2
4	18.7	16.1
6	11.4	10.1
8	12.4	7.1
12	5.1	5.4
16	< 2.5	2.5
24	< 2.5	< 2.5

system, could also be used for the determination of hydralazine and related compounds which form cyclic tetrazolo derivatives. For the analysis of complex biological extracts, the use of HPLC in a reversed-phase mode of operation ensures reasonable column life as polar materials are not retained on the column as they are during adsorption chromatography. No appreciable loss in resolution of the column was observed during the analysis of 250 plasma samples.

Zak *et al.*[11] have recently suggested that the actual levels of hydralazine measured using the derivatization procedure under acidic conditions and gas chromatography[10] were dependent on the pH of the derivatization reaction. At low pH (pH 1), measured levels may be the sum of real hydralazine concentrations plus those of postulated acid-labile "conjugate" metabolites of hydralazine, whereas at less acidic pH (pH 3), they may be real hydralazine concentrations only. Although there is no evidence at present to suggest that similar considerations may apply during the analysis of dihydralazine by the derivatization procedure and HPLC as described in this paper, the possibility is being examined further.

ACKNOWLEDGEMENTS

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